Attomole Measurement of Labeled Compounds

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Accelerator mass spectrometry (AMS) is an ultrasensitive analytical technique for measuring rare nuclides such as ^{14}C, ^{26}Al, and ^{41}Ca. The unique analytical capabilities of AMS allow long-term biological studies that cannot be performed with other methods. This paper describes the analytical capabilities of this technique and provides an overview of biomedical applications that have used AMS, including some of the work under way at the Purdue Rare Isotope Measurement Laboratory (PRIME Lab).

Description of AMS and Potential for Analysis

Introduction

Accelerator mass spectrometry (AMS) is an ultrasensitive analytical technique for measuring rare nuclides. Single atoms of one isotope can be counted in the presence of 10^{15} atoms of a neighboring isotope. AMS is used mainly for measuring radionuclides since stable nuclides are usually sufficiently abundant to measure with smaller instruments. The potential for AMS to make possible major advances in several areas of biomedicine has come to the fore in recent years. Several successful applications have been described in review articles (1-7). AMS can now be used routinely for studies that use the radionuclides ^{10}Be, ^{14}C, ^{26}Al, ^{36}Cl, ^{41}Ca, ^{129}I with several others under development. Possible studies include ADME (absorption, distribution, metabolism, and excretion), pharmacokinetics, and long-term nutritional studies. A few projects currently underway are summarized elsewhere in this paper.

Stable isotope tracers have the advantage that they do not produce radiation damage and have no disposal problems. However, the detection limit for stable isotopes is limited by their relatively high abundance in all natural materials. For example, ^{13}C is 1% abundant so that ^{13}C-labeled compounds are not detectable when diluted by about five orders of magnitude with natural carbon, since the ^{13}C from the natural carbon would overwhelm the signal from the labeled material. Natural modern ^{14}C is only about 10^{-10}% abundant and, therefore, if measured by AMS, has a far greater dynamic range than ^{13}C.

While ^{14}C has a potential dilution range of 12 orders of magnitude, the useful sensitivity is constrained on one side by the dose (limited by cost, radiation damage, and waste disposal) and on the other side by the efficiency of detection. Since AMS counts ^{14}C atoms (as opposed to the number of decay events) it has a detection efficiency 10^3-10^5 times greater than conventional decay counting. This superior detection efficiency makes AMS ideal for studies requiring low detection limits. AMS can measure attomole (1×10^{-15} moles) amounts of labeled compounds in milligram-sized samples.

The dose of a labeled compound is computed from the product of 1) the fraction that survives or is metabolized into the compound being measured, 2) the fraction that is recovered in the sample collected, 3) the efficiency for extraction from the sample, (for example, by liquid chromatography), and 4) the efficiency of the AMS measurement. For many studies, the dose can be so low that there is negligible radiation damage and no waste disposal problem. In most cases, the dose is only a fraction of the total radiation already present in the body. For example, a 160 pound adult human has 90 nCi of ^{40}K and 50 nCi of ^{14}C already present in the body. Many AMS studies require

doses of less than 10 nCi. The sample size required for an AMS measurement is only 0.5 to 4 mg. Samples as small as 1-10 µg can be studied if carrier (material that is depleted in the radioisotope to be measured) is added. For example, carrier for a ^{14}C sample would contain three orders of magnitude less ^{14}C than is usually present in the environment.

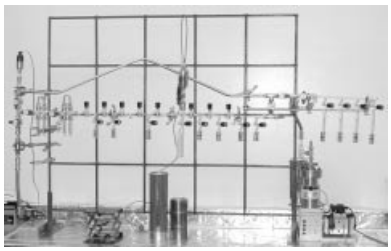
AMS Sample Preparation and Instrumentation

In this section, the techniques used at the Purdue Rare Isotope Measurement Laboratory's (PRIME Lab) AMS facility will be described. These techniques are typical of most AMS facilities.

Preparation for AMS analysis begins with the production of a compound (target material) from the sample that produces a good beam current (see the discussion of the ion source in the next paragraph) for the radionuclide to be analyzed. The production of the target material from a particular sample requires specialized chemistry. For example, the production of graphite for ^{14}C analysis involves lyophilization (or vacuum centrifugation), combustion, addition of carrier (optional), and graphitization. The vacuum line used to transfer CO_2 after combustion is shown in ***F1***. Target materials for the radionuclides analyzed by PRIME Lab are listed in ***T1***. PRIME Lab has a chemistry staff adept at making these compounds from various types of samples. The target materials are then pressed into conducting, cylindrical rods (cathodes - ***F2***). The cathodes are attached to a circular cassette (sample wheel) that holds eight cathodes. The wheel is then placed under vacuum in the ion source of the AMS.

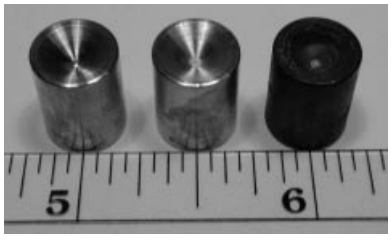
F1

The vacuum line used in the production of graphite from biomedical samples.



F2

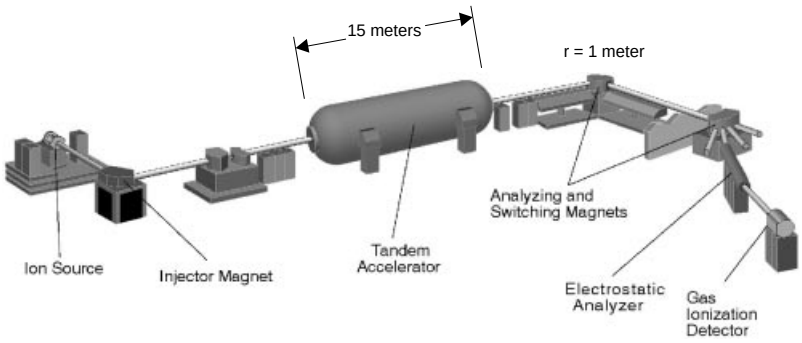
Three of the cathodes used in the cesium sputter ion source. The one on the left is empty, the cathode in the middle is filled with CaF_2, and the cathode on the right has been bombarded with cesium ions in the source. Only a few milligrams of material are needed to fill these sample holders.



The ion source focuses a cesium ion beam onto a small spot on the target material. Negative ions formed are extracted from the surface of the compound and focused into the first magnet. This 90° magnet selects the radioisotope of interest, together with any isobars (isotopes of neighboring elements and molecules that have the same mass). Most of the much more intense, stable isotopes are rejected (not bent by 90°). Most AMS systems use a tandem electrostatic accelerator consisting of two accelerating sections with a large positive voltage between them. The voltage of the accelerator used at PRIME Lab can be as high as 7.5 MV. In the positive terminal at the center of the accelerator, the negative ions are stripped of several electrons (molecular ions are dissociated into their constituent atoms) by a gas or thin carbon foil, and emerge as positive ions. These positive ions are then repelled by the terminal and accelerate to ground potential at the far end of the accelerator. The name "tandem accelerator" comes from this dual acceleration concept. After acceleration, interfering particles are further removed by an analyzing magnet, a velocity selector, a switching magnet, and an electrostatic analyzer. Finally, ions enter a gas ionization detector where they slow down and come to rest in propane gas. As the ions stop, electrons are knocked off the gas atoms. These electrons are collected onto metal plates, amplified, digitized, and read into a computer work station which deduces the nuclear charge (element atomic number) from the rate of energy loss and uses this information to differentiate between interfering isobars. A schematic of PRIME Lab's instrument is shown in ***F3***.

F3

Schematic diagram of PRIME Lab's AMS instrument.



THE PRIME Lab AMS Facility

PRIME Lab is a dedicated research and service facility for AMS. Located in the 31,000 square foot Purdue physics building sub-basement, this facility is centered around the Physics department's tandem (7.5 MV) accelerator. Currently, PRIME Lab determines the radionuclides ^{10}Be, ^{14}C, ^{26}Al, ^{36}Cl, ^{41}Ca, and ^{129}I. The target material, sample size, precision, and detection limit for each isotope are shown in ***T1***.

Recently, PRIME Lab has begun a major initiative in the biomedical sciences. Several biomedical projects that involve ^{14}C, ^{26}Al, and ^{41}Ca, are underway or have been completed. As a result of these projects, the biomedical staff has developed expertise in the preparation and handling of biological samples for AMS. A few of these studies are summa-

T1

Analytical characteristics of the Purdue PRIME Lab AMS facility.

**Precision quoted for samples above 10^{-12} atom/atom. The precision quoted is for a typical analysis. Higher precision takes more time and costs more; conversely, lower precision can reduce time requirement and cost.*

‡This detection limit is sufficient for most biomedical applications; however, work is currently on-going to improve ^{41}Ca capabilities.

Nuclide	Chemical Form	Sample size, mg	Precision, %*	Detection Limit (10^{-15})
^{10}Be	BeO	1	3	5
^{14}C	C (graphite)	0.5	1	2
^{26}Al	Al_2O_3	1	7	10
^{36}Cl	AgCl	4	3	0.5
^{41}Ca	CaF_2	1	5	1000‡
^{129}I	AgI	1	5	20

rized in the PRIME Lab Research section.

Applications

Overview

Carbon-14 is the most generally useful isotope for biomedical studies. The detection limit of AMS has made it possible to study carcinogens *in vivo* at doses that are at the environmental level (8-14). Using AMS can eliminate the need for extrapolation down to natural doses from larger doses. AMS has made it possible to trace extremely low levels of dermal absorption (15-17) of ^{14}C labeled compounds and their metabolites. AMS has also made it possible to study subjects for long periods of time, since the nuclear half-lives are long compared to biological lifetimes (18). AMS has proven utility in drug metabolism since only nCi doses of a labeled drug are needed (19). Pharmacokinetic studies were performed with a physiologic dose (35 µg, 100 nCi) of folic acid administered to a healthy, adult, human male. The study of folate metabolism is important since severe health problems (cancer and heart disease) can be associated with modest declines in dietary folate intake and when mutations in folate enzymes are present. AMS was able to follow this dose for 202 days in plasma, erythrocytes, urine, and feces. It was found that the mean lifetime of folate in the body was 93 to 120 days (20).

The possible neurotoxicity of aluminum at environmental levels has heightened interest in the ADME of aluminum. Knowledge of chemical and physiological factors that affect aluminum uptake and retention is relatively sparse. Studies with stable aluminum (^{27}Al) are not very sensitive since concentrations in the blood are very low and contamination by aluminum in the environment is a problem. Of the three longest lived isotopes ^{26}Al ($t_{1/2}$ = 716,000 y), ^{28}Al ($t_{1/2}$ = 2.2 min), and ^{29}Al ($t_{1/2}$ = 6.6 min), only ^{26}Al can be used for a study of aluminum retention over a reasonable time period. It has been shown that the radiation dose for the body only needs to be one order of magnitude lower than the daily natural dose rate for measurement by AMS (21). AMS is an invaluable tool for studying aluminum absorption and kinetics and PRIME Lab has taken a lead in this effort.

The *in vivo* absorption of ^{26}Al in aluminum-containing vaccine adjuvants has been measured (22). The biokinetics of aluminum has been explored with ^{26}Al in human subjects and compartmental modeling showed that patients with renal failure had different compartment sizes than those of healthy adults (21). The oral bioavailability of ^{26}Al has also been explored (23,24). Alzheimer's disease has been investigated by assaying the uptake of ^{26}Al into rat brains (25,26). Other studies of Alzheimer's disease have focused on the binding sites of aluminum to neuroblastoma cells. It was found that ^{26}Al did not appear to bind to DNA/RNA (27) and that gallium may be used as a surrogate for aluminum in cell culture studies (28). Use of ^{26}Al as a biological tracer is detailed in a book chapter (3).

Most of the AMS work with ^{41}Ca involves bone metabolism because of the widespread incidence of osteoporosis. The use of ^{41}Ca in conjunction with AMS provides a very sensitive means of measuring the impact of drugs and dietary changes on calcium resorption (29-31). Recently, the impact of the drugs raloxifene and alendronate on calcium resorption has been studied with ^{41}Ca. Meaningful data were obtained in a short time since AMS measurements were made with a 2% precision as opposed to 30-35% for common biomarkers (32).

PRIME Lab Research

Small Intestinal Submucosa

Professor Steve Badylak, M.D., and coworkers in the Purdue Biomedical Engineering Department have been investigating the properties of a tissue obtained from pig intestines that shows great surgical promise. This tissue, known as small intestinal submucosa (SIS), can be used for tissue replacements without rejection by the eventual host. Furthermore, the SIS material appears to be replaced by regenerated tissue over time. However, the fate of the SIS material must be determined if it is to be used with human subjects. The small intestines of three pigs were labeled by feeding them a diet enriched in ^{14}C. This produced SIS that is 10^4-10^5 times contemporary (the amount of ^{14}C currently present in the environment). This material was then placed over holes in the bladder walls of several dogs. At various time intervals tissue, blood, and other samples were converted to graphite and analyzed by AMS. Initial AMS data indicate that 90% of the ^{14}C-labeled material is gone from the bladder after approximately four weeks (33).

Cholesterol Turnover

A PRIME Lab collaboration with Richard Ostlund (Washington University, School of Medicine) explores the rate of cholesterol turnover in the body. The balance between ingested cholesterol and cholesterol that is naturally produced in the body is not well understood. The presence of cholesterol causes the formation of oxysterols (the products of cholesterol oxidation) which are of great interest since, in the crystalline form, they seem to increase clearance of cholesterol from the body. However, they have also been implicated as a possible cause of atherosclerosis. AMS is an attractive technique for the measurement of cholesterol and oxysterols. Cholesterol is present in high concentrations in the blood (2mg/mL); approximately 70 grams of cholesterol is found in the body. Thus, a tracer dose is diluted by a large factor. However, with the sensitivity of AMS, low-level tracer studies in human subjects are possible. Another consideration is that oxysterol concentrations in plasma are 3 to 4 orders of magnitude lower than cholesterol concentrations. Thus, after a few minutes, isotope ratio and gas chromatograph (GC) mass spectrometers can no longer measure tracer doses from stable isotopes. Since oxysterols are present in much lower concentrations than cholesterol, a single sample does not yield enough material to fill a cathode (*cf.*, ***F2***). Furthermore, the exact weight of the sample is unknown. Thus, isotopic dilution with enriched (98-99%) ^{13}C was explored. Results from these experiments indicate that measurements can be obtained from small biomedical samples. To evaluate the natural background and to check for contamination, samples from human subjects that had not been dosed were graphitized along with commercially produced cholesterol samples (Sigma Chemical Co.). No evidence of contamination was found. Further work is in progress to characterize this novel sample dilution technique.

Dermal absorption of aluminum-26 from antiperspirants

PRIME Lab is collaborating with Richard Flarend (Mathematics and Natural Sciences, Pennsylvania State University-Altoona) to study the dermal absorption of aluminum from antiperspirants. A small quantity of aluminum chlorohydrate (ACH), the active ingredient in antiperspirants, was labeled with ^{26}Al. The labeled antiperspirant was applied to the shaved underarms of two adult (one male and one female) subjects. After application of the labeled antiperspirant the area was covered with a large bandage. For the next six days tape strippings (masking tape applied to the underarm to collect surface ACH), bandages, and mild washings of the skin were collected for measurement. Urine and blood samples were collected from the subjects for seven weeks. Selected samples were measured at PRIME Lab. It was found that the amount of aluminum absorbed from a one-time use of antiperspirant was about 4% of the aluminum typically absorbed by the gut in a day from food. Thus, a one-time application of antiperspirant does not add a significant amount to the body's pool of aluminum (34).

Aluminum transport across a plasma membrane

Another project examines the inhibition of plant growth by aluminum. The toxic effect of aluminum is not well understood since analytical techniques sensitive enough to measure the transport of aluminum across the plasma membrane have remained elusive. However, AMS in conjunction with a unique experimental system (giant internodal cells of the alga *chara corallina*) has provided the first quanitative measurements of aluminum transport across the plasma membrane of single cells (35).

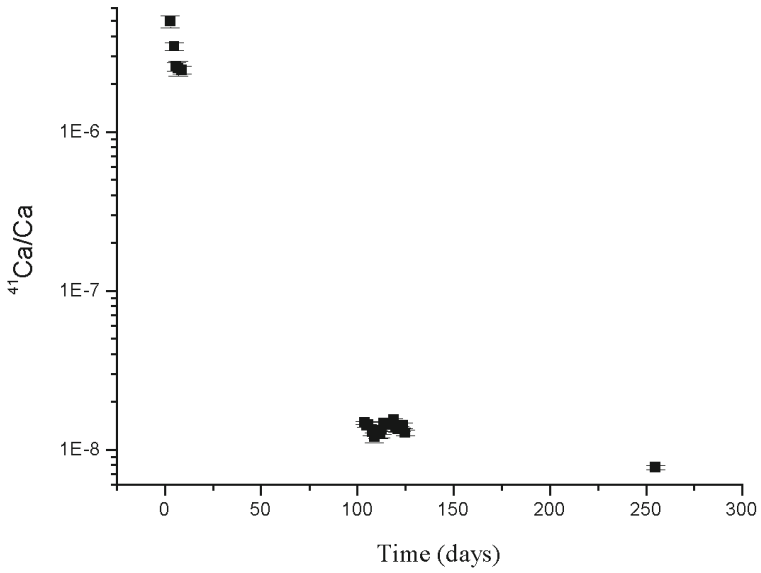
Vaccine Analysis

A collaboration has recently been initiated with Harm Hogenesch (Veterinary Pathobiology, Purdue University) and Stan Hem (Department of Industrial and Physical Pharmacy, Purdue University) that involves AMS measurements for two isotopes: ^{14}C and ^{26}Al. The overall goal of this research is to understand the mechanism by which aluminum adjuvants enhance the immune response in order to design safer and more effective vaccines.

It has been known for years that an adjuvant needs to be used in conjunction with an antigen in order to increase the immune response to a vaccine. Currently, the only adjuvant approved for use by the United States Food and Drug Administration contains aluminum. Thus, interest is great in understanding the role of the

F4

Preliminary ^{41}Ca data from urine samples. The subject was dosed with one microcurie of ^{41}Ca.



aluminum adjuvant in vaccination. For years the *depot theory* has held the fore in theories concerning the role of the aluminum adjuvant. According to this theory, the antigen is initially adsorbed to the adjuvant, then slowly released. However, this theory was contradicted by a study performed at PRIME Lab wherein ^{26}Al was detected in the blood and urine of laboratory rabbits immediately after injection (22). It is now conjectured that the main function of the aluminum adjuvant may be to accelerate the activation of dendritic cells and cause them to immediately travel down the afferent lymph vessels to the lymph node. A study involving sheep is being used to explore this theory. Surgical removal of the animal's lymph node will cause the smaller lymph vessels that drain into the lymph node to connect to the large efferent lymph vessel that carries fluid from the lymph node. The lymph may then be collected by simply cannulating the efferent lymph vessel. The sheep will be injected with vaccine that consists of an ^{26}Al-labeled adjuvant and a ^{14}C-labeled antigen (ovalbumin). The collected samples will then be analyzed for both nuclides. Issues to be addressed include whether the antigen and adjuvant stay adsorbed to each other, and whether the activation of dendritic cells is a possible mechanism by which an adjuvant enhances a vaccine's effectiveness.

Calcium Metabolism

Living bone constantly exchanges calcium with the rest of the body. This serves two purposes. First, the bone can remodel itself for optimal support throughout life. Secondly, bone contains 99% of the body's calcium and serves as a reservoir for homeostasis. Bone calcium absorption and resorption (loss from the bone) are closely linked. However, a slight imbalance in favor of resorption can cause a loss of calcium from the bone, ultimately leading to a decrease in bone mass. The difference between the amount of calcium consumed and excreted is quite small and difficult to measure. However, radioisotopes of calcium provide good tracers for these experiments. Calcium-45 provides a useful tracer for many studies. However, its short half-life (164 days) and the high doses required for liquid scintillation counting experiments are limiting. The capability of detecting ^{41}Ca ($t_{1/2}$ = 1.04 x 10^5 y) at high sensitivity with AMS has the advantage of allowing long-term studies with no radiation risk.

A collaboration with Connie Weaver (Foods and Nutrition, Purdue University) has involved the labeling of 20 post-menopausal women for long-term studies. During a study to determine the impact of soy isoflavones on calcium metabolism, these women were intravenously injected with ^{45}Ca and one μCi of ^{41}Ca. Urine samples were collected from these subjects over a period of several months. ***F4*** shows ^{41}Ca data for one of these subjects and illustrates the ability of AMS to measure data for a long period of time. Even the sample collected after 255 days is still four orders of magnitude above the background. The high ^{41}Ca/^{40}Ca ratio shown in this figure indicates that, in future experiments, we could decrease the dose by one to two orders of magnitude.

Another on-going project involves work with Elsa Janle at BAS to establish a protocol for the dosing of rats. To that end, four baby rats were dosed with eight nCi of ^{41}Ca. Calcium fluoride has been prepared from rat feces and urine. Analysis of these samples will take place soon.

Summary

The low detection limits and small sample size requirements that AMS affords are a potential boon for biomedical applications. In the last decade, the utility of this technique has just started to be realized. Small AMS instruments (36-38) could create a market for commercial instruments with the throughput and robustness needed by the biomedical community at large. PRIME Lab is well positioned to maintain its place at the cutting edge of this scientific revolution. The symbiotic partnership between PRIME Lab and BAS should prove to be a fertile one as this unique analytical technique is used to solve problems of biological importance.

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